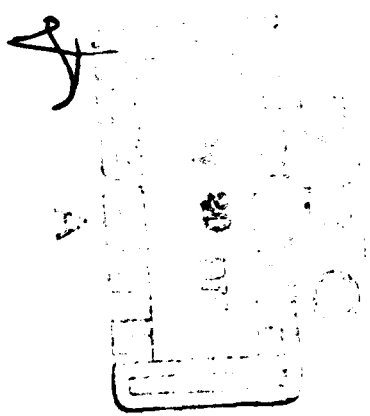

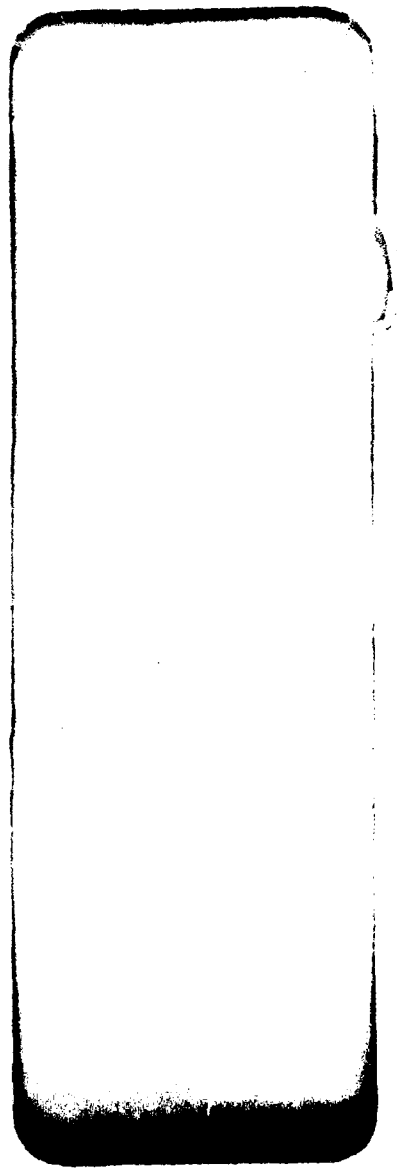

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SPECIFICITY AND IMMUNOSUPPRESSIVE POTENCY OF A RABBIT ANTIMOUSE T CELL-SPECIFIC ANTISERUM[1]

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SUMMARY

An attempt was made to prepare a specific heterologous rabbit antimouse T cell antiserum (anti-MTLA) by absorbing rabbit antimouse thymocyte globulin (ATG) with spleen cells from BALB/c T_xB_M mice. Cytotoxicity data showed that whereas ATG was cytotoxic to both T and B cells, anti-MTLA was highly cytotoxic to only T cells. Whereas spleen cells treated with ATG and complement (C) failed to respond in all assays studied, spleen cells treated with anti-MTLA and C: (1) responded to the B cell mitogens but failed to respond to the T cell mitogens; (2) were able to stimulate allogeneic spleen cells but failed to respond to mixed lymphocyte culture (3) failed to act as T killer cells in the CML reaction but retained their ability to kill antibody-coated target cells; and (4) did not cause a graft-versus-host reaction when injected in allogeneic mice and increased their survival significantly. Furthermore, anti-MTLA was just as immunosuppressive in vivo as ATG in its ability to suppress the immune response to sheep red blood cells and prolong skin allograft survival. Anti-MTLA was found to be different in specificities from anti-θ serum by several points: (1) it was cytotoxic for T cells from both θ-C3H and θ-AKR mice; (2) it was highly immunosuppressive in vivo when compared to anti-θ serum; (3) absorption of anti-MTLA with mouse brain did not decrease the immunosuppressive activity; and (4) rabbit antimouse brain antiserum failed to show any immunosuppressive activity. These data indicate that anti-MTLA is a specific antiserum against a unique marker on T cells distinct from the θ marker.

Several surface antigens have been described as being present on the surface of lymphoid cells of the mouse (3). Both thymus-derived lymphocytes (T cells) and bone marrow-derived lymphocytes (B cells) possess on their surface the major histocompatibility H-2 antigens and Ly antigens; however, certain antigens such as the TL and the theta (θ) isoantigens are present only on T cells, and the newly described β isoantigen and mouse B lymphocyte antigen (MBLA) are present only on B cells (*18–20*). The isoantisera against these markers are used in vitro to distinguish these subpopulations of lymphocytes and to further eliminate T and B cells by their cytotoxic potential (*18, 19, 22*). These in vitro treated cells demonstrate the functional reactivity expected of specific T cells or B cells depending on the reagent used. However, studies of the in vivo reactivity of such specific antisera have been few and unsuccessful. There is some controversy as to the ability of murine anti-θ serum to prolong skin graft survival. Whereas Gelfand and Paul

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(8) report successful prolongation of skin graft survival, others have shown the inability of anti-θ serum to prolong skin graft survival and to suppress the immune response to thymic-dependent antigens (15).

Various investigations have shown some degree of specificity of heterologous antisera against T or B cells in the mouse (4, 16, 18, 24) and in humans (2, 10, 26, 32). We have previously shown that antihuman thymocyte serum (ATS) absorbed with human lymphocytoblast (B cells) is just as immunosuppressive as unabsorbed ATS (23). In the same study, a high titer antiserum against lymphocytoblasts did not show any significant immunosuppressive potency. Subsequently, it was described that such absorbed sera contain an antibody specific for human T cells (HTLA) (26). These sera did not have any cytotoxic reactivity against human lymphocytoblasts, and the cytotoxic titer against human thymocytes correlated with allograft prolongation (27).

The purpose of this investigation was to determine the cell surface antigen requirement for producing a similar heterologous rabbit antimouse T cell-specific antiserum by absorption of heterologous antithymocyte globulin (ATG) with murine B cells. Furthermore, the purpose was to define the various in vitro and in vivo functions of T and B cells after treatment with such antisera in the presence of complement.

MATERIALS AND METHODS

Animals. Mice of the inbred strains AKR/J, BALB/c, C57BL/6J, and C3H/HeJ (3–6 weeks old) were obtained from The Jackson Laboratory, Bar Harbor, Maine. New Zealand White rabbits (3 kg) were obtained from Rowmar Rabbitory, Mt. Airy, Maryland. Nu/Nu and Nu/+ littermates (Swiss strain) were a generous donation of Dr. A. D. Steinberg and Dr. E. Gershwin of the National Institutes of Health, Bethesda, Maryland.

Preparation of rabbit antimouse thymocyte globulin (ATG). ATG was prepared by the method described previously (14). BALB/c thymocytes (1×10^9) incorporated in Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan) were injected in the footpads of rabbits, followed 2 weeks later with a similar booster injection by the i.m. route. Two weeks after the last injection, the rabbits were exsanguinated and the serum was collected. The γG fraction was obtained by precipitation of the serum with 33% saturated ammonium sulfate. The precipitate was dissolved and dialyzed against several changes of 0.15 M NaCl and concentrated by the use of an Amicon (UM-2) membrane filter (Amicon Corporation, Bedford, Massachusetts) so as to contain 5 mg of protein/ml. Normal rabbit γ-globulin (NγG), 5 mg/ml, was prepared in a similar fashion. Both ATG and NγG were stored in aliquots at 70 C until further use.

Anti-serum. Mouse anti-θ C3H was prepared by the immunization of AKR/J mice with C3H/HeJ thymocytes according to the procedure of Reif and Allen (19) as modified by Strong et al. (28).

Rabbit antimouse brain γ-globulin (RAMB). RAMB antisera were prepared according to the procedure outlined by Golub (9). The γG fraction was prepared as described above and had a 50% cytotoxicity titer of 1:4,000.

Preparation of rabbit antimouse T cell-specific γ-globulin. Fifty milliliters of ATG (5 mg/ml) were absorbed with 1 g of mouse liver powder (Difco) at 4 C for 1 hr with gentle rotation. The suspension was centrifuged at 750 *g* for 30 min and the ATG was removed by aspiration. This ATG was absorbed with 'B' cells. Spleen cells from BALB/c mice which were thymectomized, irradiated, and bone marrow (anti-θ + C treated)-reconstituted (T_xB_M) were used as a source of 'B' cells. ATG (1 ml) was incubated with 10×10^8 B cells either once (designated absorbed ATG-I) or twice (designated absorbed ATG-II) at 22 C for 30 min followed by an additional incubation at 4 C for 30 min. After incubation, the suspension was centrifuged at 450 *g* for 15 min and the absorbed ATG was removed and used in the various assay systems.

Preparations of T_xB_M spleen cells used as B cells were checked for purity by the ability of these cells to respond to the nonspecific T cell mitogens phytohemagglutinin (PHA-P) and concanavalin A (Con A) (28) and the B cell mitogens bacterial lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid (poly I.C.) (22). These cells were also identified and enumerated by staining for surface immunoglobulin. Results of these experiments indicated complete lack of response of these cells to the T cell mitogens and that > 90% of

the cells used for ATG absorption showed B cell surface immunoglobulin characteristics.

Absorption of ATG with mouse brain. ATG was absorbed with BALB/c brain according to the method of Reif and Allen (*19*).

Cytotoxicity assay. The cytotoxic activity of the various antisera used during these studies was assayed by the trypan blue dye exclusion method and the ^{51}Cr release method in the presence of rabbit complement (C). The dye exclusion test was performed according to the method of Takahashi et al. (*30*). Essentially, 0.05 ml of cells ($5 \cdot 10^6$ ml) was incubated for 45 min at 37 C with 0.05 ml of antiserum (serially diluted) in the presence of 0.05 ml of rabbit complement (C) (preabsorbed with mouse liver powder) diluted 1:3. After the incubation period, 0.1 ml of 0.9% trypan blue was added and the percentage of viability was detemined.

The chromium release assay was performed according to the methods of Sanderson (*21*) and Wigzell (35) with some modifications. Spleen cells were incubated with NH_4Cl to remove red blood cells and the resulting lymphoid cells were labeled with 300 μc of ^{51}Cr sodium chromate (Amersham Searle, Arlington Heights, Illinois). Labeled spleen cells were resuspended in media such that 50 μl contained 1×10^6 cells. These were dispensed in microtiter plates and 50 μl of serially diluted antisera were added to triplicate cultures. These cultures were incubated at 4 C for 30 min, and then 50 μl of C diluted 1:4 were added to each well and the microtiter plate was shaken on a micromixer and incubated at 37 C for 30 min. Controls included NγG, C, and media control. After the incubation period, the plates were centrifuged and 100 μl were removed and counted in a γ counter for ^{51}Cr. One hundred per cent release of ^{51}Cr was determined by the freeze-thaw lysis of $1 \cdot 10^6$ spleen cells. The percentage of ^{51}Cr release was calculated as previously described (*1*).

In vitro stimulation assay. RPMI 1640 (Grand Island Biological Company, Grand Island, New York) containing 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 25 mM Hepes, and 10% heat-inactivated fetal calf serum (Grand Island) was used as media throughout these studies. One milliliter of murine spleen cells, $20 \cdot 10^6$/ml, was treated with 0.2 ml of either ATG, absorbed ATG, or NγG in the presence of 0.1 ml of undiluted rabbit C. The mixture was incubated at 37 C for 45 min. The suspension was washed and resuspended in 2.5 ml of media so as to give $8 \cdot 10^6$ spleen cells/ml and used for the mitogen stimulation test. The mitogen stimulation test was performed by methods previously described (*22, 28*). PHA-P and Con A were used as T cell mitogens and LPS and poly I:C were used as B cell mitogens. Optimum concentrations of mitogen and spleen cells and culture conditions were determined by baseline experiments. PHA-P (Difco) was used at a final concentration of 0.1%; Con A (Calbiochem, San Diego, California) at a concentration of 0.25 μg/culture; LPS (Difco 0111:B4) at a concentration of 20 μg/culture; and poly I:C (P-L Biochemicals, Milwaukee, Wisconsin) at a concentration of 50 μg/culture. Cultures were performed in triplicate at 37 C in a 5% CO_2 humidified atmosphere. The cultures were pulsed with 1 μc of ^3H-thymidine (^3H-TdR) (specific activity 1.9 c/m mole, Schwarz-Mann, Orangeburg, New York) in 20 μl of media 18 hr before harvest. The mean uptake of ^3H-TdR was determined at 66 hr.

Mixed lymphocyte culture (MLC) assay. The one-way micro-MLC assay was performed as previously described (*31*). Spleen cells were treated with antisera and C as described for the mitogen study. Spleen cells from BALB/c and C3H/HeJ mice were used both as responder and stimulator cells. The stimulator cells were treated with 50 μg/ml of mitomycin C (Calbiochem) before culture. The cultures contained 100 μl ($5 \cdot 10^5$ cells) of responder cells and 100 μl ($5 \cdot 10^5$) of stimulator cells. They were labeled with 1 μc of ^3H-TdR in 20 μl of media 18 hr before harvest. They were harvested at 5 days and the mean cpm uptake of ^3H-TdR was calculated.

Lymphocyte-mediated cytotoxicity assay (CML). C57BL/6J mice were sensitized in vivo by the administration of $20 \cdot 10^6$ BALB/c spleen cells i.p. L-1210 mouse ascites tumor cells were carried in DBA/2 mice and used as target cells. Target cells were labeled with ^{51}Cr as described above. The CML assay was performed in microtiter plates by the addition of 100 μl of effector cells (which were either untreated or treated with ATG or absorbed ATG-II and C as described above) to 10^4 target cells in the ratio of 100:1. Red cells were removed by the addition of NH_4Cl. The cul-

tures were mixed on a microtiter plate mixer (Cooke Engineering, Bethesda, Maryland) and the amount of ^{51}Cr release was determined after 16 hr of incubation as described above.

Antibody-dependent lymphocytotoxicity assay (ADL). Normal C57BL/6J spleen cells (untreated, ATG-, or absorbed ATG-II-treated) were depleted of red cells by the addition of NH_4Cl and used as effector cells in the ADL assay. ^{51}Cr-labeled L-1210 cells ($5 \cdot 10^6$/ml) were incubated with 0.1 ml of anti-H-2^d antisera (obtained through the courtesy of the Transplantation Immunology Branch, National Institute of Allergy and Infectous Diseases Maryland) for 1 hr at 4 C and used as target cells. The effector to target cell ratio was 50:1 and consisted of $6 \cdot 10^4$ target cells and 6×10^6 effector cells. The amount of ^{51}Cr released was determined after 4 hr of incubation, as described above, and the net percentage of ^{51}Cr release was calculated.

Graft-versus-host (GVH) reaction assay. GVH reaction is an example of a cell-mediated immune response (T cells). It is further shown that two types of thymus-derived lymphocytes, T_1 and T_2, cooperate to produce GVH (5). In our assay, mixtures of 50×10^6 spleen cells and 5×10^6 bone marrow cells from C57BL/6J mice were treated with 0.5 ml of either NγG, ATG, or absorbed ATG-II in the presence of rabbit C. Subsequently, these cells were washed and injected i.v. into lethally irradiated (750 R) BALB/c mice. Groups of four mice each were used for the in vitro GVH assay by the method of Strong et al. (29), measuring spontaneous uptake of ^3H-TdR in vitro by the recipient BALB/c spleen cells on day 4 after injection of allogeneic cells. Groups of 10 mice each were used for the GVH survival assay. The survival rate of these irradiated, allogeneic cell-injected mice was followed for a period of 12 weeks.

Plaque-forming assay. BALB/c mice received a s.c. injection of various doses of either ATG, absorbed ATG-I, RAMB, ATG absorbed with mouse brain, or anti-θ C3H sera. One day later they received $2 \cdot 10^8$ sheep red blood cells (SRBC) (Microbiological Associates) via the i.p. route. Four days after sensitization, their spleens were removed and the number of anti-SRBC antibody-forming cells was estimated by the hemolytic plaque method of Cunningham and Szenberg (7). Direct plaque-forming cells (D-PFC) were measured 4 days after sensitization and indirect PFC (I-PFC) was developed by the addition of a 1:100 dilution of rabbit antimouse IgG (a gift from Dr. M. Kern, National Institutes of Health) at day 7. The number of I-PFC was determined by subtracting the number of D-PFC from the total PFC developed with anti-IgG.

Skin grafting. BALB/c mice were grafted with whole split-thickness skin from C57BL/6J mice according to the technique described previously (13). Groups of BALB/c recipient mice received four s.c. injections of either NγG, ATG, absorbed ATG-I, or anti-θ C3H sera 1 day before and on days 1, 3, and 5 after skin grafting. The casts were removed after 7 days and the skin grafts were examined daily for signs of rejection. The data were collected on the time required for complete rejection and statistical analysis of the data was analyzed using the Mann-Whitney 'U' ranking test (25).

RESULTS

The cytotoxic activity of the various antisera against T and B cells used in these studies was determined by the trypan blue dye exclusion technique. BALB/c thymocytes were used as a source of T cells and T_XB_M spleen cells from BALB/c mice were used as a source of B cells. As seen in Figure 1, unabsorbed ATG was cytotoxic for both T and B cells. When this ATG was absorbed with 10×10^8 T_XB_M spleen cells (ATG-I), the 50% cytotoxic titer against T cells essentially remained the same, but the titer against B cells was reduced from 1:512 to 1:2. On a second absorption of ATG-I with 10×10^8 T_XB_M spleen cells (ATG-II), there was a reduction in titer against T cells decreasing from 1:4,000 to 1:1,000 and a further decrease in the titer against B cells. This decrease in titer against T cells could be attributable to the few contaminating T cells in T_XB_M spleen cells. Anti-θ C3H was used as an index of purity of the B target cells and, as seen in Figure 1, anti-θ C3H shows about 10% cytotoxicity against T_XB_M spleen cells when used undiluted, indicating few contaminating T cells.

When a similar cytotoxicity assay was carried out using ^{51}Cr-labeled Nu/+ and Nu/Nu spleen cells, it was determined again that after absorption of ATG with T_XB_M spleen cells (ATG-I) there is no reduction in the cytotoxic titer against Nu/+, but a considerable decrease

in the cytotoxic titer against Nu/Nu spleen cells (Fig. 2).

In order to test the functional capacity of lymphoid cells treated with ATG, ATG-I, or ATG-II, spleen cells treated with these antisera and C were incubated with the T cell mitogens PHA-P and Con A or the B cell mitogens LPS and poly I:C. As seen in Figure 3, ATG completely abolished the response to both the T cell mitogens and the B cell mitogens. However, ATG-I, while retaining its cytotoxic activity against T cells, had a slight effect on the response to B cell mitogens. This effect on B cell mitogen response was completely removed by one more absorption of ATG-I with T_xB_M spleen cells (ATG-II). This data, therefore, shows that ATG-I and II retain their activity against T cells, whereas they have little or no action on the ability of treated spleen cells to respond to B cell mitogens. To determine whether ATG-II was reactive against markers other than θ and H-2, similar mitogen assays were carried out using cells from AKR/J, C3H/HeJ, and C57BL/6J mice. As seen in Figure 4, spleen cells from all of these strains of mice which were treated with ATG-II and C, while showing adequate response to the B cell mitogens LPS and poly I:C, failed to respond to the T cell

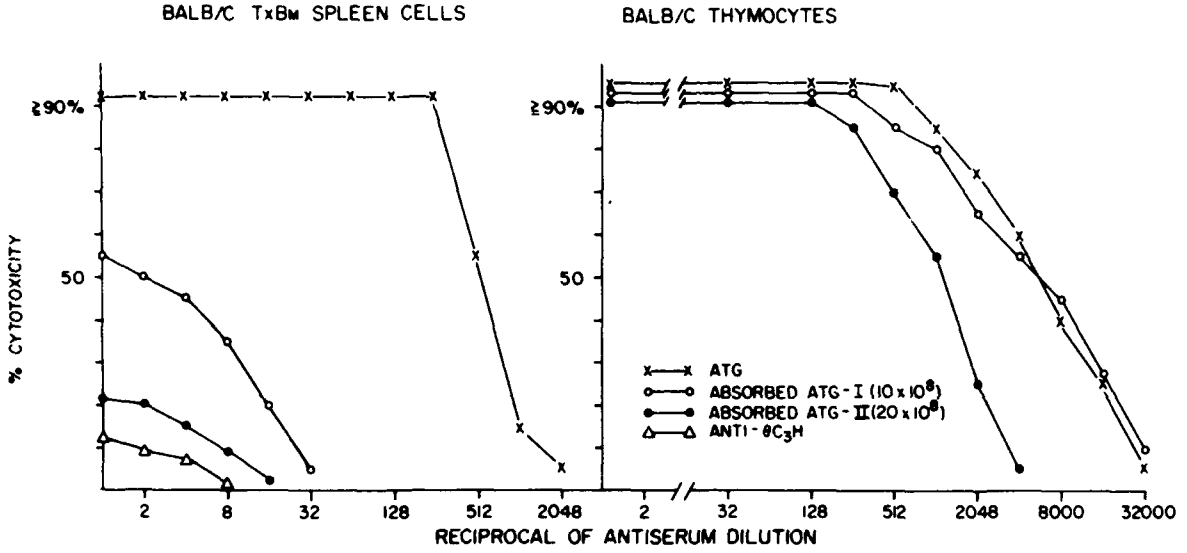

FIG. 1. Cytotoxic titer of ATG, absorbed ATG-I, and ATG-II as determined by the dye exclusion method using BALB/c thymocytes and BALB/c T_xB_M spleen cells as targets for T and B cells.

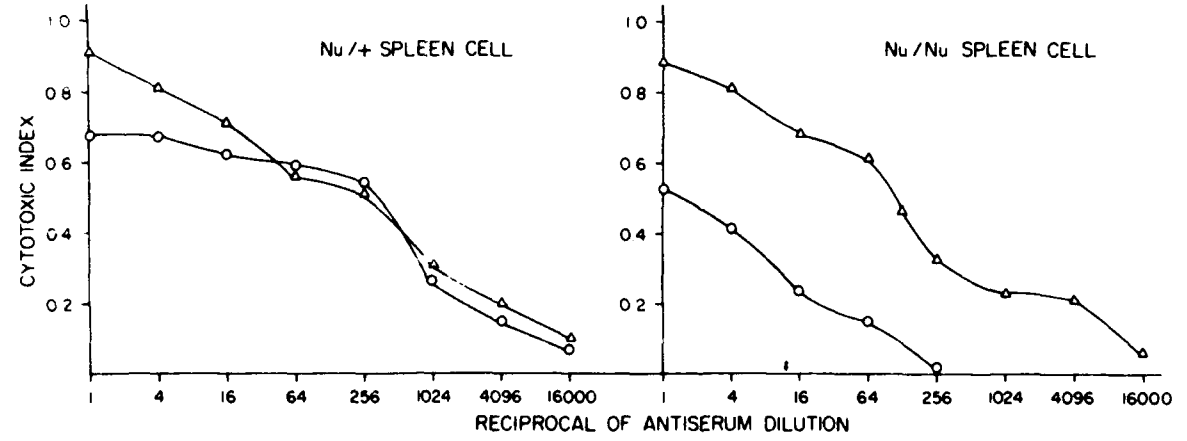

FIG. 2. Cytotoxic titer of ATG and absorbed ATG-I as determined by the chromium release assay using spleen cells from Nu/Nu (B cells) and Nu/+ (both T and B cells) as target cells. ATG, Δ——Δ; ATG-I, O——O.

$$\text{Cytotoxic index} = \frac{\text{Mean net cpm of experimental values}}{\text{Mean net cpm of freeze-thaw control}}$$

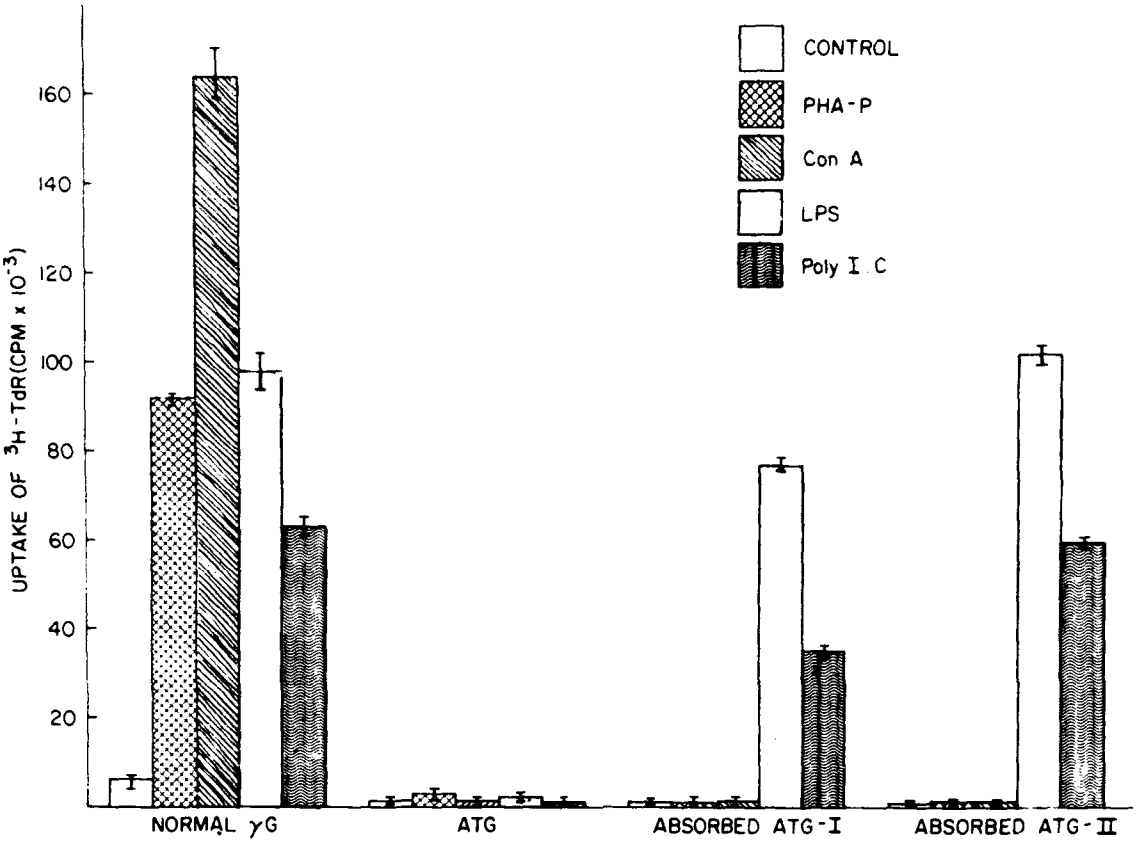

FIG. 3. Response of spleen cells treated with NγG, ATG, absorbed ATG-I, and absorbed ATG-II to the nonspecific T cell mitogens PHA-P and Con A and the B cell mitogens LPS and poly I:C.

mitogens PHA and Con A. Experiments with ATG-II and C-treated spleen cells from C3H mice were repeated five times and essentially gave similar results. Absorption of ATG-II with mouse brain while decreasing the titer against mouse thymocytes still retained its ability to eliminate the response of spleen cells to both PHA and Con A, but not to LPS and poly I:C. This pointed to the fact that antibodies in ATG-II were directed against surface antigens other than θ or H-2.

Because T cells are necessary in the responder population during MLC and B cells can act as stimulator cells in the MLC reaction, spleen cells from C3H/HeJ mice were treated with ATG, absorbed ATG-II, or conventional anti-θ serum in the presence of C and subsequently used as responder cells against BALB/c stimulator cells. As seen in Table 1, both ATG and ATG-II completely inhibited the ability of C3H/HeJ cells to respond to BALB/c spleen cells. On the other hand, when BALB/c stimulator cells were treated with ATG or ATG-II or anti-θ C3H and C, it is seen (Table 2) that whereas ATG completely inhibited the ability of BALB/c spleen cells to act as stimulator cells, both anti-θ and ATG-II did not affect the ability of these BALB/c spleen cells to stimulate allogeneic C3H/HeJ spleen cells.

Another parameter of T cell function is the ability of sensitized T cells to kill allogeneic target cells. Spleen cells from C57BL/6J mice which were sensitized in vivo by the injection of allogeneic BALB/c spleen cells were used in a direct lymphocytotoxicity assay using ^{51}Cr-labeled L-1210 mouse ascites tumor cells as target cells. The L-1210 cells carry the same serologically defined H-2 specificities as the BALB/c spleen cells. The effector cells were either treated with ATG, ATG-II, or anti-θ serum. Controls consisted of untreated, NγG, or normal mouse serum (NMS)-treated effector cells. As seen in Table 3, ATG-, ATG-II, and anti-θ-treated spleen cells reduced the capacity of the effector cells to kill target cells, whereas NγG, NMS-treated, and untreated spleen cells showed a net percentage of ^{51}Cr release of about 30%.

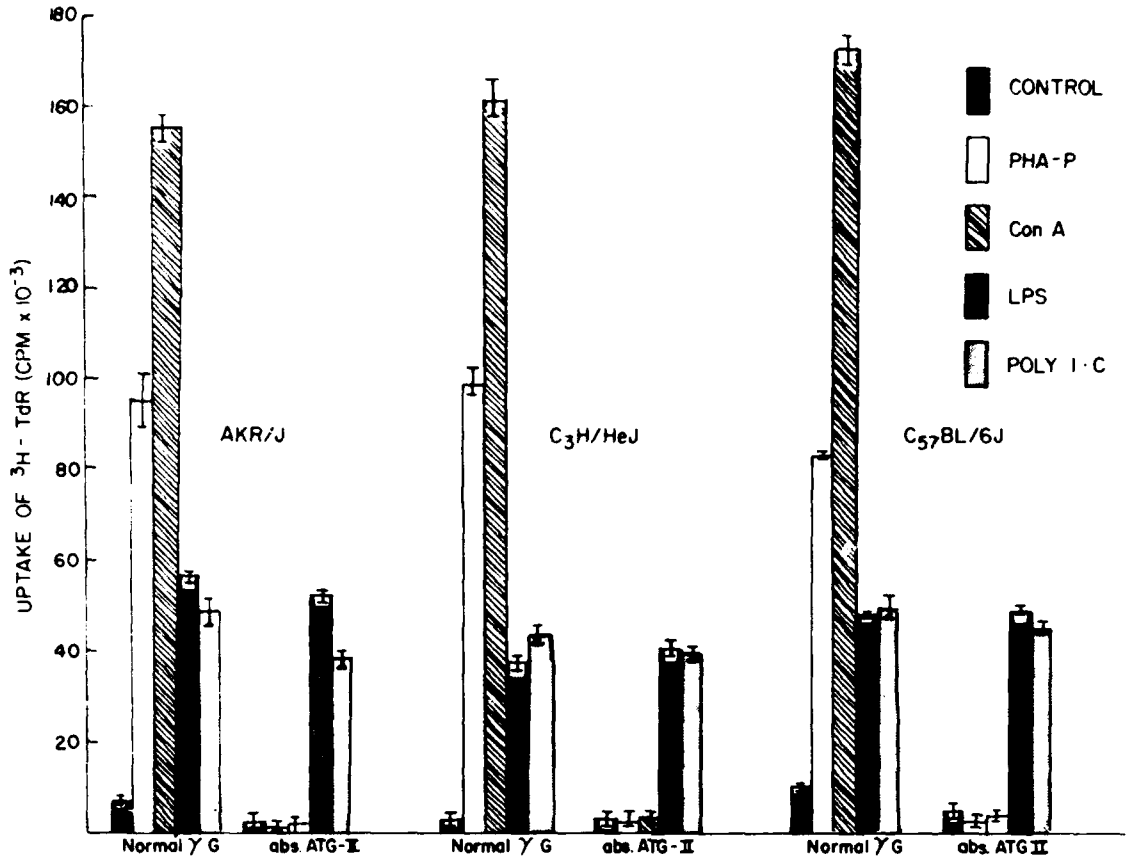

FIG. 4. Response of spleen cells from AKR/J, C57BL/6J, and C3H/HeJ mice treated with NγG or absorbed ATG-II to the nonspecific T cell mitogens PHA-P and Con A and the B cell mitogens LPS and poly I:C.

TABLE 1. Ability of C3H/HeJ spleen cells to respond to allogeneic cells in the MLC reaction after treatment with ATG or absorbed ATG and C

Stimulator[a]	C3H/HeJ responder pretreated with[b]	Mean uptake of ^{3}H-TdR (cpm ± SE)
—	—	2,498.6 ± 88.1
—	Normal rabbit γG	1,822.0 ± 37.2
—	Normal mouse serum	1,440.3 ± 32.3
—	ATG	522.0 ± 22.1
—	Absorbed ATG-II	1,294.3 ± 28.1
—	Anti-θ	889.6 ± 22.4
BALB/c	—	15,167.3 ± 199.1
BALB/c	Normal rabbit γG	18,937.3 ± 305.7
BALB/c	Normal mouse serum	12,299.3 ± 160.6
BALB/c	ATG	678.3 ± 10.9
BALB/c	Absorbed ATG-II	1,237.0 ± 60.4
BALB/c	Anti-θ	863.0 ± 21.3
BALB/c control	—	200.3 ± 8.4

[a] Stimulator BALB/c spleen cells were treated with 50 μg/ml of mitomycin C at 37 C for 45 min.

[b] Spleen cells, 5×10^5/culture, were used as stimulator or responder cells.

Normal lymphoid cells other than T cells possess the capacity of killing target cells when the target cells are coated with antisera directed against the target cells (the antibody-dependent lymphocytotoxicity assay). To determine the specificity of ATG-II, experiments were carried out where normal C57BL/6J spleen cells were either treated with ATG or ATG-II or anti-θ serum and C. Again, controls consisted of untreated C57BL/6J spleen cells or NγG or NMS and C-treated C57BL/6J spleen cells. As seen in Table 4, spleen cells treated with unabsorbed ATG showed no reactivity against antibody-coated L-1210 cells. However, when spleen cells were treated with anti-θ serum or ATG-II, there was little effect on the cytotoxic ability of these cells (56 to 31 and 38%, respectively). NMS- and NγG-treated spleen cells also showed a decrease from 56 to about 46%. These data and the CML data indicate that whereas ATG-II destroys the T cytotoxic cells, it has very little effect on B cells (or non-T cells), which are necessary for antibody-dependent B cell cytotoxicity.

TABLE 2. Ability of BALB/c spleen cells to stimulate allogeneic cells in the MLC reaction after treatment with ATG or absorbed ATG and C

BALB/c stimulator cells treated with[a, b]	Responder[b]	Mean uptake of ^{3}H-TdR (cpm ± SE)
—	—	200.3 ± 8.4
Normal rabbit γG	—	218.0 ± 18.5
Normal mouse serum	—	144.6 ± 27.4
ATG	—	238.6 ± 10.7
Absorbed ATG-II	—	166.6 ± 9.7
Anti-θ	—	258.0 ± 7.8
—	C3H/HeJ	15,167.3 ± 199.1
Normal rabbit γG	C3H/HeJ	17,286.0 ± 1,318.3
Normal mouse serum	C3H/HeJ	15,185.0 ± 181.8
ATG	C3H/HeJ	710.0 ± 26.7
Absorbed ATG-II	C3H/HeJ	12,480.0 ± 682.8
Anti-θ	C3H/HeJ	9,491.0 ± 210.1
	C3H/HeJ control	2,498.6 ± 88.1

[a] Stimulator BALB/c spleen cells were treated with 50 μg/ml of mitomycin C at 37 C for 45 min.

[b] Spleen cells, 5×10^5/culture, were used as stimulator or responder cells.

TABLE 3. CML reactivity of sensitized C57BL/6J spleen cells[a]

Treatment of effector cells[b]	Net chromium release[c] (%)
Normal mouse serum	32.87
Anti-θ C3H	4.64
Normal rabbit γG	34.82
ATG	2.99
Absorbed ATG-II	1.90

[a] C57BL/6J mice were sensitized with BALB/c spleen cells and ^{51}Cr-labeled L-1210 ascites tumor cells were used as target cells.

[b] Spleen cells (20×10^6) were treated with 0.1 ml of antiserum and C.

[c] Per cent chromium release was determined as described in the text.

The ability to cause a GVH reaction is thought to be a function of T cells. To determine if cells treated with whole ATG or absorbed ATG-II were able to induce a GVH reaction, spleen cells from C57BL/6J mice were either treated with NγG or ATG or absorbed ATG-II in the presence of C. Subsequently, these cells were injected into lethally irradiated BALB/c recipients and the degree of a GVH reaction was determined by the method of Strong et al. (29). As seen in Table 5, spleen cells treated with ATG or ATG-II gave no significant GVH reactivity (ratios of 1.43 and 4.28, respectively), but the NγG-treated and untreated C57BL/6J spleen cells gave activation ratios of 52.45 and 90.37, respectively.

The survival rate of these recipient BALB/c mice was followed and, as seen in Figure 5, X-irradiated animals (group I) all died between 2 and 3 weeks. Mice receiving cells treated with NγG (group II) died within 2 weeks post-reconstitution, which was earlier than the non-treated group 1. Group III received cells treated with ATG, and 90% of these animals died within 4 weeks of reconstitution; however, 60% of group IV animals receiving cells treated with ATG-II lived > 12 weeks postreconstitution.

To test the in vivo immunosuppressive potency of ATG or absorbed ATG-I, mice were immunized with an i.v. injection of 2×10^8 SRBC. Twenty-four hours before immunization, groups of mice received either NγG, ATG, or ATG-I. D-PFC were determined on day 4 after immunization and I-PFC were determined on day 7 after immunization by subtracting the number of direct PFC from the total PFC. As seen in Figures 6 and 7, ATG suppressed both the direct and indirect PFC response of BALB/c mice against SRBC and the degree of suppression correlated with doses of ATG injected. It is also clearly shown that absorbed ATG-I revealed the same degree of immunosuppressive potency as ATG in both responses.

To determine whether anti-θ C3H and RAMB had similar immunosuppressive activity, experiments were carried out where groups of

TABLE 4. ADL reactivity of C57BL/6J spleen cells treated with ATG and C

Treatment[a]	Net chromium release (L-1210 target cells)[b] (%)
—	55.98
Normal mouse serum	45.80
Anti-θ C3H	30.92
Normal rabbit γG	46.22
ATG	0.74
Absorbed ATG-II	37.48

[a] Normal C57BL/6J spleen cells (20×10^6) were treated with 0.1 ml of antiserum and C.

[b] Anti-H-2^d-coated ^{51}Cr-labeled L-1210 mouse ascites cells were used as target cells.

TABLE 5. GVH reactivity of C57BL/6J spleen cells treated with ATG and C in recipient BALB/c mice

Donor strain	Treatment[a]	Spontaneous activation of recipient spleen cells (mean cpm ± SE)	Stimulation index
—[b]	—	321 ± 157	—
BALB/c	—	1,677 ± 436	5.22
C57BL/6J	—	29,008 ± 2,306	90.37
C57BL/6J	Normal rabbit γG	16,835 ± 1,827	52.45
C57BL/6J	ATG	458 ± 79	1.43
C57BL/6J	Absorbed ATG-II	1,373 ± 652	4.28

[a] GVH reactivity was measured as described in the text. Spleen cells (50×10^6) and bone marrow cells (5×10^6) from C57BL/6J mice were pretreated with 0.5 ml of antiserum and C.

[b] No cells were injected into X-irradiated BALB/c mice.

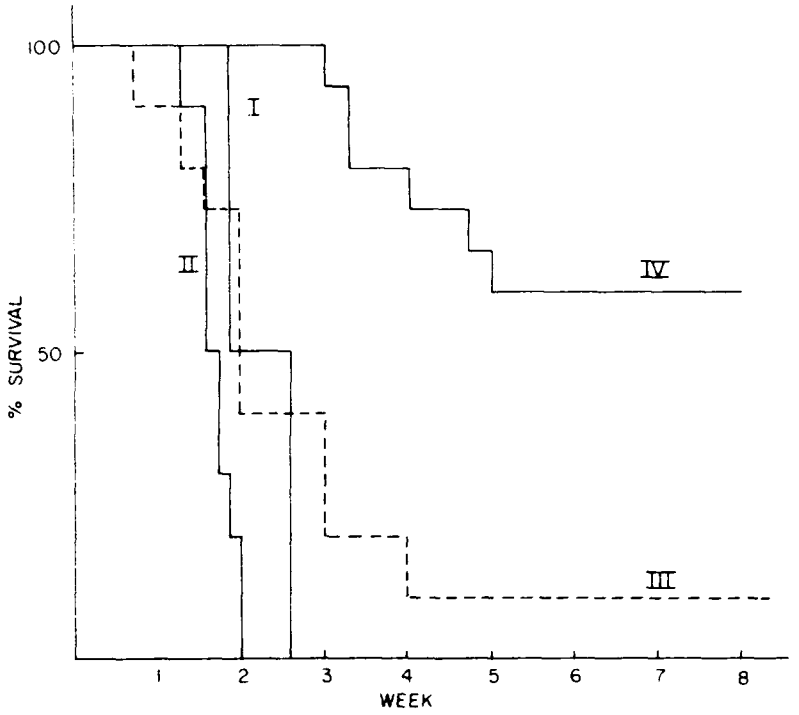

FIG. 5. Survival rate of lethally irradiated BALB/c mice reconstituted with C57BL/6J spleen and bone marrow cells pretreated either with NγG, (group II), ATG (group III) or absorbed ATG-II (group IV). Group I received no cells after irradiation.

mice received 0.5 ml each of either anti-θ C3H, NγG, or RAMB 24 hr before sensitization with SRBC. As seen in Table 6, anti-θ C3H and RAMB did not show any immunosuppressive activity when compared to the effect of NγG. On the other hand, ATG, ATG-I, and ATG absorbed with mouse brain decreased both the D-PFC and the I-PFC significantly.

ATG is also known to prolong skin graft survival by its in vivo immunosuppressive potency. Groups of BALB/c mice were injected either with NγG, ATG, ATG-I, anti-θ C3H, or RAMB according to the protocol described in Materials and Methods. Twenty-four hours later they were grafted with skin from C57BL/6J mice and the rejection time was determined. As seen in Table 7, the mean skin graft survival time of the mouse injected with NγG was 10.1 days. ATG administration prolonged the survival from 10.1 to 24.8 days ($P < 0.001$) and ATG-I prolonged the survival time from 10.1 to 23.6 days ($P < 0.001$). Anti-θ C3H showed very little in vivo immunosuppressive potency (mean survival time 11.7 ± 0.8) and, similarly, RAMB was not immunosuppressive (10.3 ± 0.7 and 11.1 ± 0.9). On

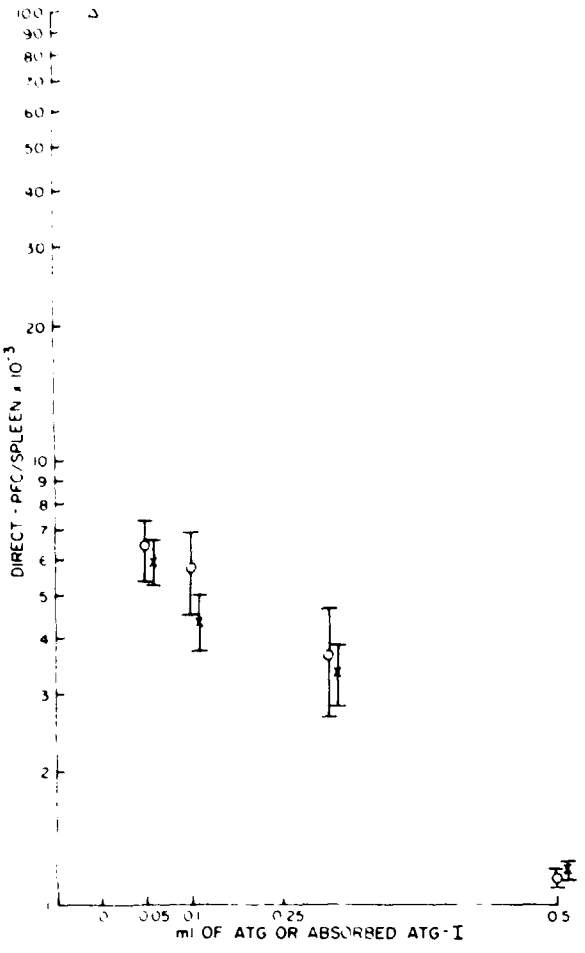

FIG. 6. Effect of ATG or absorbed ATG-I on the direct PFC response against SRBC in the BALB/c mice. ATG or absorbed ATG-I was injected 1 day before sensitization and the PFC response was measured 4 days after sensitization. x, ATG; O, absorbed ATG-I; Δ, no injection.

the other hand, ATG absorbed with mouse brain prolonged skin graft survival time (22.3 ± 0.9). There was no statistical difference between the immunosuppressive potency of ATG or ATG-I.

DISCUSSION

T and B cells are distinguished either functionally or by the presence of unique surface antigens and receptors on their cell surface (*12, 17*). Some of the well characterized surface antigens include the histocompatibility antigens HL-A in man and H-2 in mice, the minor H antigens, the thymus-leukemia antigens TL, Ly, MSLA, θ, and MBLA in the mouse, and HTLA in the human (*2-4, 10, 18-20, 24, 26, 32*). The preparation of monospecific antisera against these markers provides for a unique biological means for the study of the role of T and B cells in the immune system. ATS is a well known potent immunosuppressive agent because of its effect on T cells (*11*), but the site against which the immunosuppressive antibody is directed remains unclear. The data presented here show that a "heterologous" rabbit antimouse thymocyte serum is cytotoxic for both T cells and B cells, whereas after absorption with spleen cells from T_xB_M mice these absorbed antisera (ATG-II) retain all their properties against T cells but do not appear to have any effect on B cells.

Whereas spleen cells when treated with ATG + C failed to respond to both the T cell mitogens (PHA-P and Con A) and the B cell

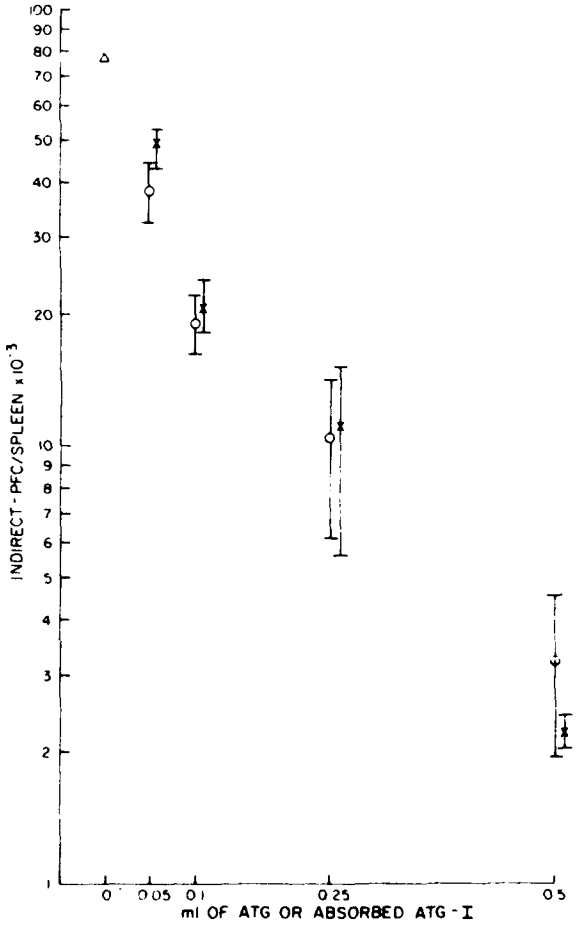

FIG. 7. Effect of ATG or absorbed ATG-I on the indirect PFC response against SRBC in the BALB/c mice. ATG or absorbed ATG-I was injected 1 day before sensitization and the PFC response was measured 7 days after sensitization. x, ATG; O, absorbed ATG-I; Δ, no injection.

mitogens (LPS and poly I:C), absorbed ATG + C-treated spleen cells failed to respond only to the T cell mitogens. These data indicate that the cytotoxicity in absorbed ATG-I and II is specific for T cells. MLC studies with cells treated with such absorbed sera and C showed that although such cells failed to respond in a MLC against allogeneic cells, they were able to stimulate just as well as NMS-treated cells. On the other hand, ATG + C-treated spleen cells were unable to stimulate or respond in the MLC. Because it has been shown that T cells are required for responder cells but not for stimulator cells (*31*), this clearly demonstrated that the absorbed ATG is only cytotoxic for T cells. Another function of T cells is to kill target cells to which it has been previously sensitized (*6*). B cells, macrophages, or null cells, on the other hand, kill target cells only if the target cells are coated with antibody directed against themselves (*33*). When spleen cells from sensitized mice were treated with absorbed ATG and C, these cells fail to kill allogeneic target cells, but there was no effect on the ability of these cells to kill antibody-coated target cells; this, again, demonstrates its T cell-specific cytotoxic activity.

The in vitro treatment of lymphoid cells with absorbed ATG reduced the capacity of immunocomponent cells to cause a GVH reaction. When lethally irradiated BALB/c mice were reconstituted with normal spleen cells from C57BL/6J mice, these animals died within 2–3 weeks. Ninety per cent of the mice receiving ATG-treated spleen cells died within 4 weeks. However, 60% of absorbed ATG-treated spleen cells survived up to 12 weeks. The data indicate that whereas ATG has some protective role, absorbed ATG is certainly far superior in the prolongation of life of lethally irradiated reconstituted mice. This is probably attributable to its ability to specifically kill GVH-causing cells without a notable effect on B cells. Results with the in vivo studies showed that administration of this absorbed antisera (ATG-I) was just as immunosuppressive as ATG in its ability to reduce both direct and indirect PFC, prolonging skin graft survival just as effectively as ATG.

The data in this paper indicate that when mouse thymocytes are used as a source of antigen for the production of ATG in rabbits (a heterologous species), the antisera contain activity against both T and B cells, which indicates some shared antigens between T and B cells which the rabbit can recognize and produce antibodies against. After absorption of this ATG with B cells with a short incubation period, this antisera showed specificity for T cells demonstrating a specific T cell antigen which the rabbit can recognize. This antibody shows activity in vitro which is very similar to that of conventional anti-θ serum. However, it is functionally different from anti-θ in that: (1) it

TABLE 6. Effect of various antisera on the primary immune response of BALB/c mice against SRBC

Antisera[a]	D-PFC/spleen ± SE[b]	I-PFC/spleen ± SE[c]
No treatment	101,563 ± 13,532	78,653 ± 12,631
Normal rabbit γG	54,166 ± 4,811	49,831 ± 4,870
ATG	1,171 ± 30	2,280 ± 62
Absorbed ATG-I	1,120 ± 20	3,225 ± 1,312
ATG absorbed with brain	2,351 ± 831	4,836 ± 529
Rabbit antimouse brain γG	89,959 ± 7,826	54,381 ± 8,976
Anti-θ C3H	68,748 ± 5,231	48,572 ± 6,730

[a] Antiserum (0.5 ml) was injected 1 day before sensitization.
[b] Direct PFC/spleen was measured 4 days after sensitization.
[c] Indirect PFC/spleen was determined on day 7 by subtracting the number of D-PFC from the total PFC.

TABLE 7. Effect of in vivo administration of ATG on skin graft survival time (C57BL/6J → BALB/c)

Treatment[a]	No. of animals	Mean survival time (days ± SE)	Range
Normal rabbit γG	15	10.1 ± 0.3	9–11
Rabbit γG antimouse brain (batch I)	12	10.3 ± 0.7	9–12
Rabbit γG antimouse brain (batch II)	11	11.1 ± 0.9	9–13
ATG	11	24.8 ± 0.5	21–28
Absorbed ATG-I	5	23.6 ± 0.8	21–25
ATG absorbed with mouse brain	5	22.3 ± 0.9	19–27
Anti-θ C3H	4	11.7 ± 0.8	7–13

[a] Recipient BALB/c mice were injected with 0.5 ml of antiserum on days −1, +1, +3, and +5.

was cytotoxic for T cells from both θ-C3H and θ-AKR mice (Fig. 4); and (2) whereas absorbed ATG was just as immunosuppressive as ATG in its ability to suppress the humoral immune response and prolong allogeneic graft survival, anti-θ serum had been shown to be nonimmunosuppressive in vivo.

This absorbed ATG is also different from the RAMB in that the latter is not immunosuppressive even though its cytotoxic titer is just as high as anti-θ or ATG. These studies indicate that the immunosuppressive antibody is not against the brain θ antigen and is not an antispecies antibody (heteroantisera). Also, when ATG was absorbed with brain it did not lose its immunosuppressive potency, further emphasizing that the immunosuppressive property of ATG is not directed against the θ markers. These data indicate that T cells have on their surface a specific differentiation antigen which is not found on other cells. It appears that absorbed ATG is directed against such an antigen and that cells bearing this marker are responsible for the various T cell functions described in these studies. Therefore, we propose to call this antisera anti-mouse T cell-specific antisera (anti-MTLA).

Another heteroantisera against mouse lymphocytes has been described by Shigeno et al. (24). They termed it anti-MSLA (mouse-specific lymphocyte antigen). This antisera was prepared with the in vivo absorption of ATS in mice, and the main purpose of this research was to produce antisera specific for lymphoid cells and not other tissues. They also found that ATS did not contain antibody against other well known surface antigens such as TL, Ly-A, Ly-B, or θ, but did contain low titer antibody against H-2 antigens (24). This antisera, however, was not specific for either T cells or B cells.

Our studies extend the previous observations of Sell et al. (23) that anti-T cell antibodies are the immunosuppressive antibodies in ATG and not the antilymphoblast or anti-B cell antibodies. These data also agree with those of Smith and Woody (27), who showed that the cytotoxic titer of HTLA, a specific rabbit anti-human T cell antisera against thymocytes, correlated very well with its in vivo immunosuppressive effect.

If ATG, according to Shigeno et al. (24), does not contain antibodies to TL, Ly-A, Ly-B, or θ, then our data suggest that the antigen against which the absorbed ATG is directed is not H-2, is not present on B cells, is specific for T cells, and can be recognized by another species. Development of such antisera against specific subpopulations of T cells, which cause allograft rejection, may lead to specific immunosuppression without effects on other cells.

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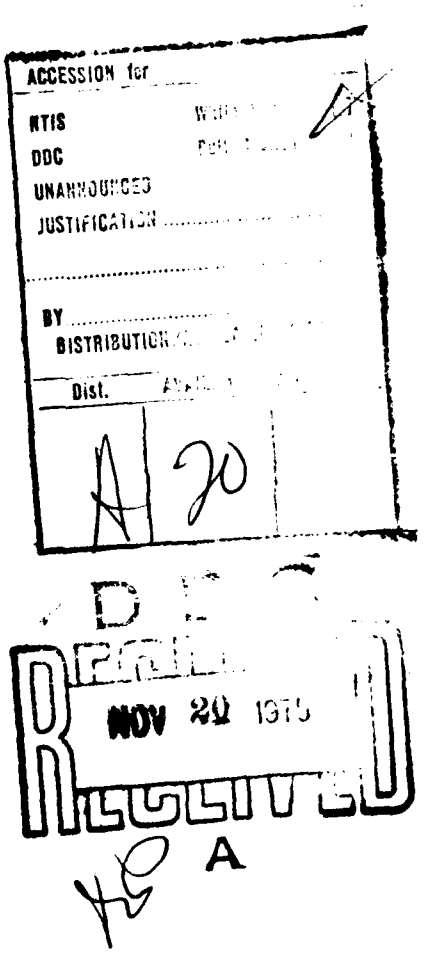

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SPECIFICITY AND IMMUNOSUPPRESSIVE POTENCY OF A RABBIT ANTIMOUSE T CELL-SPECIFIC ANTISERUM

MEDICAL RESEARCH PROGRESS REPORT

TAKENORI OCHIAI, AFTAB AHMED, DOUGLAS M. STRONG, IRWIN SCHER, KENNETH W. SELL

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An attempt was made to prepare a specific heterologous rabbit antimouse T cell antiserum (anti-MTLA) by absorbing rabbit antimouse thymocyte globulin (ATG) with spleen cells from BALB/c T_XB_M mice. Cytotoxicity data showed that whereas ATG was cytotoxic to both T and B cells, anti-MTLA was highly cytotoxic to only T cells. Whereas spleen cells treated with ATG and complement (C) failed to respond in all assays studied, spleen cells treated with anti-MTLA and C: (1) responded to the B cell mitogens but failed to respond to the T cell mitogens; (2) were able to stimulate allogeneic spleen cells but failed to respond in mixed lymphocyte culture; (3) failed to act as T killer cells in the CML reaction but retained their ability to kill antibody-coated target cells; and (4) did not cause graft-versus-host reaction when injected in allogeneic mice and increased their survival significantly. Furthermore, anti-MTLA was just as immunosuppressive in vivo as ATG in its ability to suppress the immune response to sheep red blood cells and prolong skin allograft survival. Anti-MTLA was found to be different in specificities from anti-θ serum by several points: (1) it was cytotoxic for T cells from both θ-C3H and θ-AKR mice; (2) it was highly immunosuppressive in vivo when compared to anti-θ serum; (3) absorption of anti-MTLA with mouse brain did not decrease the immunosuppressive activity; and (4) rabbit antimouse brain antiserum failed to show any immunosuppressive activity. These data indicate that anti-MTLA is a specific antiserum against a unique marker on T cells distinct from the θ marker.

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